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(21) International Application Number: PCT/US92/08467 (22) International Filing Date: 5 October 1992 (05.10.92) (30) Priority data: 770,608 3 October 1991 (03.10.91) US (71) Applicant: DANA-FARBER CANCER INSTITUTE, INC. [US/US]; 44 Binney Street, Boston, MA 02115 (US). (72) Inventors: TEDDER, Thomas, F. ; Five Indian Ridge Way, South Natick, MA 01760-5627 (US). SPERTINI, Olivier, G. ; Route de Malipalud, CH-1042 Assens (CH). (74) Agents: GAGNEBIN, Charles, L., III et al. ; Weingarten, Schurgin, Gagnebin & Hayes, Ten Post Office Square, Boston, MA 02109 (US).		(81) Designated States: AU, CA, JP. Published <i>With international search report.</i>
(54) Title: LEUKOCYTE ADHESION MOLECULE-1 (LAM-1) AND LIGAND THEREOF (57) Abstract A leukocyte-associated cell surface protein LAM-1 (leukocyte adhesion molecule-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins; the specific domains of the LAM-1 protein; ligand binding fragments of the LAM-1 protein; and the genomic DNA sequences encoding the LAM-1 protein and the specific domains of LAM-1 are disclosed. Also disclosed are methods and agents for detecting, identifying and characterizing the LAM-1 ligand. The LAM-1 protein or a ligand binding fragment thereof or an antagonist to the LAM-1 protein or ligand binding fragment thereof are used in methods of detecting sites of inflammation or disease in a human patient. They are also used in therapeutic compositions in methods of treating a patient suffering from a leukocyte-mobilizing condition.		

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LEUKOCYTE ADHESION MOLECULE-1
(LAM-1) AND LIGAND THEREOF

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This application is a continuation-in-part of Tedder, U.S. Patent Application Serial No. 07/700,773, filed May 15, 1991, the whole of which is hereby incorporated by reference herein.

Part of the work leading to this invention was made with United States Government funds. Therefore, the U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

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This invention relates to human leukocyte-associated cell surface proteins and particularly to leukocyte adhesion molecule-1 (LAM-1).

BACKGROUND OF THE INVENTION

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Genes exclusively expressed by one cell lineage, but not by others, often define the function of that cell population. The generation of genes by the assembly of functionally independent domains has occurred frequently as new genes have evolved to encode proteins with new functions. An inducible endothelial-leukocyte adhesion molecule (ELAM-1), having several functionally independent domains, is expressed on the surface of cytokine-treated endothelial cells. This molecule is thought to be responsible for the accumulation of blood leukocytes at sites of inflammation by mediating the adhesion of cells to the vascular lining (Bevilacqua et al., Proc. Natl. Acad. Sci. USA 84:9238 (1987)). A granule membrane protein found in platelets and endothelial cells, termed GMP-140, has been cloned and is homologous with ELAM-1 (Johnston et al., Blood Suppl. 1 72:327A (1988)).

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SUMMARY OF THE INVENTION

The invention generally features a leukocyte-associated cell surface protein LAM-1 (leukocyte adhesion molecule-1), which contains domains homologous with binding domains of animal lectins, growth factors, and C3/C4 binding proteins; the specific domains of the LAM-1 protein; the genomic DNA sequences encoding the LAM-1 protein and the specific domains of LAM-1; methods of detecting the presence of a LAM-1 ligand; ligand binding fragments of LAM-1; methods of developing an antagonist to LAM-1 or LAM-1 ligand function; and methods of treating a human patient suffering from a leukocyte-mobilizing condition.

In one aspect the invention features a method of detecting a site of inflammation or a diseased state in a human patient that includes administering to the patient a pharmaceutical composition comprising a detectable amount of a labeled LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance, and detecting the label on the LAM-1 protein.

In preferred embodiments the LAM-1 protein, or ligand binding fragment, is labeled with a radionuclide, a paramagnetic isotope, or a radiopaque label. The LAM-1 protein, or ligand binding fragment, can also be joined to a carrier protein, preferably an immunoglobulin heavy chain constant region or non-ligand binding portions of a selectin molecule.

In another aspect the invention features an imaging agent for imaging a site of inflammation or a diseased state in a human patient that includes LAM-1 protein or a ligand binding fragment thereof labeled with a detectable label and suspended in a pharmaceutically acceptable carrier substance. In preferred embodiments the detectable label comprises a radionuclide, a paramagnetic isotope, or a radiopaque label. The LAM-1 protein, or ligand binding fragment, in the imaging agent can also be joined to a carrier protein, preferably an immunoglobulin heavy chain constant region or non-ligand binding portions of a selectin molecule.

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In another aspect the invention features a method of treating a human patient suffering from a leukocyte-mobilizing condition that includes administering to the patient a therapeutic composition comprising a therapeutic amount of LAM-1 protein or ligand binding fragment, or antagonist to the LAM-1 protein or specific ligand binding fragment, in a pharmaceutically acceptable carrier substance. Preferably, the patient is suffering from cancer, tissue damage or an autoimmune disorder. Alternatively, the patient is an organ or tissue transplant recipient. In preferred embodiments the LAM-1 protein, or ligand binding fragment, is joined to a therapeutic agent, which is preferably either a chemotherapeutic drug or an anti-inflammatory agent. Alternatively, the method further includes administering to the patient a therapeutic amount of a cell surface molecule other than LAM-1 protein or ligand binding fragment thereof, or a soluble fragment of the cell surface molecule. (The cell surface molecules or soluble fragments are those capable of participating in the adhesion of leukocytes to endothelial surfaces.)

As used herein the term "antagonist to LAM-1" includes any agent which interacts with LAM-1 and interferes with its function, e.g., antibody reactive with LAM-1 or any ligand which binds to LAM-1. The term "identify" is intended to include other activities that require identification of an entity, such as isolation or purification. The term "essentially purified" refers to a protein or nucleic acid sequence that has been separated or isolated from the environment in which it was prepared or in which it naturally occurs.

Leukocyte-associated cell surface protein LAM-1 plays an important role in leukocyte-endothelial cell interactions, especially selective cell trafficking to sites of inflammation. The LAM-1 protein or ligand binding fragments or specific domains thereof, or other molecules that interfere with leukocyte adhesion and function, can be used

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therapeutically to inhibit the inflammatory response and to treat such conditions as tissue damage and metastasis of cancer cells. The assays for LAM-1 function are highly reproducible, easily performed, and allow the precise quantitation of the extent of the LAM-1 interaction with ligand or ligands. Thus, they permit quantitation of the extent of involvement of different receptor systems in adhesion and the easy identification of antagonists to LAM-1 function.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the drawings, Figs. 1A and 1B show the structure of LAM-1 cDNA clone;

Fig. 2 shows the cDNA nucleotide sequence and also shows the amino acid sequence of LAM-1;

Figs. 3A, 3B, and 3C show the homologies of LAM-1 with other proteins;

Figs. 4A, 4B, and 4C show the restriction map and the exon-intron organization of the *lyam-1* gene; and

Fig. 5 shows the nucleotide sequence of exons II through X of the *lyam-1* gene.

The leukocyte adhesion molecule-1 (LAM-1), also called lymphocyte-associated molecule-1, is expressed by human lymphocytes, neutrophils, monocytes and their precursors and is a member of the selectin family of cellular adhesion/homing receptors which play important roles in leukocyte-endothelial cell interactions, especially selective cell trafficking to sites of inflammation. LAM-1 combines unrelated domains found in three distinct families of molecules: animal lectins, growth factors, and C3/C4 binding proteins.

A ligand for LAM-1, induced on cultured human endothelium following activation with specific inflammatory

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cytokines, is involved in the initial attachment of homing leukocytes to the endothelial cells.

Identification and characterization of the LAM-1 protein.

5 cDNA encoding the LAM-1 protein was initially identified as follows. B cell-specific cDNAs were isolated from a human tonsil cDNA library (ATCC #37546) using differential hybridization with labeled cDNAs derived from either B cell (RAJI) RNA or T cell (HSB-2) RNA (Tedder et al., Proc. Natl. Acad. Sci. USA 85:208-212 (1988)). Positive plaques were
10 isolated and cloned, and the cDNA inserts were subcloned into the plasmid pSP65 (Promega, Madison, WI). Nucleotide sequences were determined using the method of Maxam and Gilbert (Meth. Enzymol. 65:499 (1980)). Gap penalties of -1 were assessed during homology analysis for each nucleotide or amino acid in the sequence where a gap or deletion
15 occurred. One of the 261 RAJI+ HSB2- cDNA clones isolated, B125, contained a 1.90 kilo base pair (kb) cDNA insert that hybridized with a 2.4 kb RNA species found in several B cell lines (Tedder et al., supra). However, B125 did not
20 hybridize with any of the other RAJI+ RSB2- clones or with mRNA from several T cell lines. The B125 cDNA clone was characterized by restriction mapping and nucleotide sequence determination. A near-full-length 2.3 kb cDNA that hybridized with B125 was isolated, sequenced, and termed
25 pLAM-1.

The expression of LAM-1 mRNA by cell lines of lymphoid and non-lymphoid origin was examined. Northern blot analysis revealed that LAM-1 cDNA hybridized strongly to a 2.6 kb RNA species and weakly to a 1.7 kb RNA species in poly(A)+ RNA
30 isolated from the B cell lines RAJI, SB, Laz-509, and GK-5. However, RNA isolated from two pre-B cell lines (Nalm-6, PB-697), three B cell lines (Namalwa, Daudi, BJAB), five T cell lines (CEM, Hut-78, HSB-2, Molt-15, Molt-3), a myelomonocytic cell line (U937 and U937 cultured with LPS) and
35 erythroleukemic K-562 cell line did not hybridize with LAM-1

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cdNA suggesting that expression of this gene was preferentially associated with B lymphocytes. Neutrophils expressed LAM-1 mRNA but had a relatively lower amount of transcript among total mRNA when compared with the RAJI cell line or blood T lymphocytes. LAM-1 cDNA has also been used to transfer expression of LAM-1 to cells that do not express the gene.

As shown in Fig. 1A, a restriction map was constructed by the standard single, double or triple digestions of pLAM-1. The coding region is shown in black. Arrows indicate the direction and extent of nucleotide sequence determination and the open circles indicate 5'-end labeling. In Fig. 1B, a schematic model of the structure of the LAM-1 mRNA is shown, thin lines indicate 5' and 3' untranslated sequences (UT), while the thick bar indicates the translated region. The boxes represent the lectin-like and epidermal growth factor (EGF)-like domains and the two short consensus repeat (SCR) units. The open box indicates the transmembrane (TM) region.

pLAM-1 contains an open reading frame that could encode a protein of 372 amino acids as shown in Fig. 2. The numbers shown above the amino acid sequence designate amino acid residue positions. The numbers to the right indicate nucleotide residue positions. Amino acids are designated by the single-letter code, and * indicates the termination codon. The boxed sequences identify possible N-linked glycosylation sites. Hydrophobic regions that may identify signal and transmembrane peptides are underlined. The amino acid sequence of LAM-1 indicates a structure typical of a membrane glycoprotein. The mature LAM-1 protein has an extracellular region of about 294 amino acids containing 7 potential N-linked carbohydrate attachment sites. LAM-1 has a cytoplasmic tail of 17 amino acids containing 8 basic and 1 acidic residues. The processed LAM-1 protein has a Mr of at least 50,000 and can be isolated by conventional techniques, such as affinity column chromatography with antibody or ligand, from cell lines that normally express

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this receptor or from transfected cell lines. Or the protein can be synthesized by in vitro translation of the LAM-1 cDNA.

LAM-1 combines domains homologous to domains found in three distinct families of molecules: animal lectins, growth factors, and C3/C4 binding proteins. The extracellular region of LAM-1 contains a high number of Cys residues (7%) with a general structure as diagrammed in Fig. 1B. As indicated in Fig. 3, segments of homologous proteins are shown with the amino acid residue numbers at each end. Homologous amino acids are shown in boxes. Gaps (-) have been inserted in the sequences to maximize homologies. The first 157 amino acids of the protein (Fig. 3A) were homologous with the low-affinity receptor for IgE (Kikutani et al., Cell 47:657 (1986)), the asialoglycoprotein receptor (Spiess et al., Proc. Natl. Acad. Sci. USA 82:6465 (1985)) and several other carbohydrate-binding proteins (Drickamer et al., J. Biol. Chem. 256:5827 (1981); Ezekowitz et al., J. Exp. Med. 167:1034 (1988); Krusius et al., J. Biol. Chem. 262:13120-13125 (1987); and Takahashi et al., J. Biol. Chem. 260:12228 (1985)). The amino acids conserved among all animal-lectin carbohydrate recognition domains are indicated (*). Although the sequence homologies were less than 30%, all the invariant residues found in animal-lectin carbohydrate-recognition domains were conserved (Drickamer, J. Biol. Chem. 263:9557 (1988)). The lectin domain included amino acid residues 42-170 given in Fig. 2.

The next domain of 36 amino acids, at residues 171-206 shown in Fig. 2, was homologous (36-39%) with epidermal growth factor (EGF) (Gregory, Nature 257:325 (1975)) and the EGF-like repeat units found in Factor IX (Yoshitake et al., Biochem. 25:3736 (1985)) and fibroblast proteoglycan core protein (Krusius et al., supra) (Fig. 3B).

Immediately following these domains were two tandem domains of 62 amino acids each, given by residues 207-269 and 270-331 of Fig. 2, that were homologous with the short consensus repeat unit (SCR) that comprises the IL-2 receptor

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(Leonard et al., Nature 311:626 (1984)), Factor XIII (Ichinose et al., Biochem. 25:4633 (1986)) and many C3/C4 binding proteins (Klickstein et al., J. Exp. Med. 165:1095 (1987); and Morley et al., EMBO J. 3:153 (1984)). In contrast with all of the previously described SCR that contain four conserved Cys residues, these two SCR possessed six Cys residues. The four conserved Cys residues found in all SCR are indicated in Fig. 3C by (*); the additional conserved Cys found in LAM-1 are indicated by (+). Of the multiple SCR present in each of these proteins, the SCR with the highest homology to LAM-1 is diagrammed (Fig. 3C). A 15 amino acid spacer followed the short consensus repeat units, preceding the transmembrane domain.

The structure of the *lyam-1* gene, which encodes the LAM-1 protein, was determined by isolating overlapping genomic DNA clones that hybridized with a LAM-1 cDNA probe. The *lyam-1* gene spans greater than 30 kb of DNA and is composed of at least 10 exons. The 5' end of the LAM-1 mRNA was mapped by primer extension analysis, revealing a single initiation region for transcription. Exons II through X contain translated sequences; exon II encodes the translation initiation codon, residue 14 shown in Fig. 2; exon III encodes the leader peptide domain, residues 15-41; exon IV encodes the lectin-like domain, residues 42-170; exon V encodes the epidermal growth factor-like domain, residues 171-206; exons VI and VII encode the short consensus repeat unit domains, residues 207-269 and 270-331; exon VIII encodes the transmembrane region, residues 332-373; exon IX encodes seven amino acids containing a potential phosphorylation site, residues 374-380; and exon X encodes the five remaining amino acids of the cytoplasmic tail and the long 3' untranslated region.

The pLAM-1 cDNA was labeled with ³²P and used as a probe to isolate hybridizing DNAs from a human leukocyte genomic DNA library. Approximately 1 X 10⁶ plaques were screened, and 13 plaques that hybridized with the cDNA probe were

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identified and isolated. Seven of these clones were found to contain inserts with unique restriction enzyme maps representing overlapping genomic fragments spanning at least 30 kb. These inserts, LAMG-17, -19, -20, -28, -35, -37, and -47, were further digested and subcloned into plasmids. Detailed restriction maps of these subclones were made and compared to those of intact inserts to determine their correct locations (Figs. 4A and 4B).

The correctness of the restriction map was verified with Southern blot analysis. DNA isolated from two B cell lines, BL and BJAB, and one T cell line, HSB-2, was digested to completion with *Bam* HI, *Bal* II, or *Pvu* II, size-fractionated, and transferred onto nitrocellulose. This filter was probed with the LAM-1 cDNA clone, pLAM-1. All genomic fragments derived from endonuclease digested DNA hybridized with cDNA probe to generate hybridizing bands of the appropriate size.

The pLAM-1 cDNA clone encodes an 85-bp 5' untranslated region. An oligonucleotide homologous with the 5' sequence of the pLAM-1 cDNA was used as a probe for primer extension analysis. This oligonucleotide was hybridized with poly (A⁺) RNA isolated from the human B cell line RAJI, the LAM-1 negative human B cell line Namalwa, the mouse pre-B cell line A20, and yeast tRNA as a control. Complementary DNA was synthesized by extending the primer with reverse transcriptase. The major primer extension product obtained using the human LAM-1 positive B cell line RNA was extended 126 nucleotides beyond the translation initiation site. There was a single cluster of transcription initiation sites for the *lyam-1* gene apparent in the reaction with RAJI RNA that was not found with the LAM-1 negative Namalwa RNA. Several primer extension products of size similar to those of the human B cell line RNA were obtained with mouse B cell RNA. Therefore, murine B cells may express an RNA species that cross-hybridizes with the oligonucleotide probe used. No primer extension products were obtained in the yeast tRNA control reactions.

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The relationship of the primer extension results to the cloned LAM-1 cDNAs and the most 5' exon of *lyam-1* isolated was used to determine the nucleotide sequence of the exon that encodes the translation initiation AUG codon. This exon ends immediately after the site that encodes the translation initiation codon (Fig. 5) and overlaps precisely with the pLAM-1 cDNA sequence. The length of the cDNA clone obtained by Bowen et al., J. Cell Biol. 109:421-427 (1989) agrees precisely with the primer extension results except for two nucleotides. However, 15 nucleotides before the 5' end of the cDNA the sequence diverges from the genomic sequence at a site homologous with the 3' splice acceptor site consensus sequence. Therefore, it is most likely that this 15-bp region is derived from the exon upstream of this potential splice site. Thus, the primer extension results indicate that exon I would most likely be composed of 15 or fewer base pairs.

A 15-bp oligonucleotide homologous with the 5' nucleotides present in the cDNA clone of Bowen et al., supra but not encoded by exon II, was used to probe the 10 kb of cloned DNAs 5' of exon II; however, specific hybridization was not detected by Southern blot analysis. Under the conditions necessary for hybridization of this oligonucleotide, significant cross-hybridization occurred with λ -DNA, making it difficult to use this oligonucleotide to isolate the first exon from a λ -based genomic library.

These results suggest that the exon which encodes the translation initiation site is the second exon of the *lyam-1* gene (Fig. 4C). Consistent with this, the 900 bp upstream of exon II did not contain any apparent "TATA" or "CCAAT" sequences frequently found in promoter regions of eukaryotic genes (Fig. 5). Therefore, it is likely that the transcription initiation region and exon I are further than 10 kb upstream from exon II of the *lyam-1* gene. S1 nuclease protection analysis was carried out using the 5' region of exon II as a labeled probe for hybridization with poly (A⁺)

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RNA from RAJI, Namalwa, and A20 cells. Two mRNA species were protected in the RAJI mRNA, while no S1 protection was provided by the other RNAs. The length of these fragments was consistent with differential splicing at the two potential CAG/N splice sites located within the potential splice acceptor site in exon II. It is therefore likely that the transcription initiation region has not been identified.

The majority of the exons were localized by comparison of the restriction enzyme maps of the genomic clones and the pLAM-1 cDNA. In cases where this method did not provide definitive results, subcloned DNA fragments were digested with selected restriction enzymes, electrophoresed through agarose gels, and transferred to nitrocellulose. Fragments containing exons were identified by Southern blot analysis using labeled cDNA or oligonucleotide probes. The exon that encodes the 3' untranslated region of the LAM-1 cDNA was not contained within the 30 kb of isolated DNA fragments. Therefore, a labeled 0.9-kb *Dra* I fragment containing most of the 3' untranslated region of the pLAM-1 cDNA was used as a probe to identify a homologous 3.2-kb fragment generated by complete *Eco* RI digestion of genomic DNA. *Eco* RI-digested genomic DNA fragments of this size were used to make a partial λ -gt11 genomic library from which the 3.2-kb *Eco* RI fragment was cloned. This 3.2-kb fragment did not overlap with the previously isolated genomic DNAs.

The exact boundaries of the exons were determined by nucleotide sequence analysis. From this analysis, nine exons were identified which make up the entire pLAM-1 cDNA. Exon II encodes the translation initiation codon, and exon III encodes the leader domain of the LAM-1 protein (Fig. 5). Each of the lectin-like, epidermal growth factor-like, transmembrane, and short consensus repeat domains was encoded by a separate exon. The smallest exon, IX, is 19 bp in length and may encode a carboxyl-terminal phosphorylation cassette. The last 5 amino acids of the LAM-1 protein and the 3' untranslated region which includes the poly(A)

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attachment site, AATAAA, are encoded by exon X as shown in Fig. 5. The nine exons which encode pLAM-1 were split inside codons in all cases, except the junction between exons II and III. In each instance, the consensus sequences of 5' donor splice sites and 3' acceptor splice sites were adhered to. Nucleotide sequence polymorphisms within the coding region were observed between the genomic clones containing exon V that encoded SCR I and the pLAM-1 clone at cDNA nucleotide positions 741 and 747 (A to G), leading to a coding change from Asn to Ser in both cases, and at position 816 (A to G) changing the Glu to a Gly.

Detection, identification and characterization of the LAM-1 ligand.

MATERIALS AND METHODS

Endothelial cell cultures. Human umbilical vein endothelial cells (HUVEC) were isolated from two to five umbilical cord veins, pooled and established as primary cultures in medium 199 (M199, Gibco BRL, Grand Island, NY) containing 20% FCS (Gibco BRL). HUVEC were serially passaged and maintained using M199 supplemented with 10% FCS, endothelial cell growth factor (50 µ/ml, Biomedical Technologies, Inc., Stoughton, MA) and porcine intestinal heparin (50 µg/ml; Sigma, St. Louis, MO) in tissue culture flasks precoated with 0.1% gelatin. For experimental use, passage 2 to 3 of HUVEC were plated on microscopic slides coated with 0.1% gelatin and growth within a 2.2 cm diameter circle delineated by a ring of 12 M polysiloxane.

Leukocyte isolation. Blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from normal volunteers. Neutrophils were isolated by centrifugation on Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA) followed by lysis of red blood cells with ice-cold hypotonic 0.2% (w/v) NaCl solution. Cells were kept on ice until use.

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Mab and PPME. Murine monoclonal antibodies (mAb) used were those directed against distinct epitopes of LAM-1, anti-LAM1-1, -3, -4, -5, -6, -7, -8, -10 and -11, all of the IgG₁ isotype, and were used as diluted ascites fluid (1:100) (Spertini et al., Leukemia 5:300 (1991); Spertini et al., J. Immunol. (in press) (1991)). The anti-ELAM-1 mAb H18/7 (IgG₂), which inhibits the adhesion of neutrophils to activated endothelium, was used as purified F(ab')₂ fragments (25 µg/ml) (Bevilacqua et al., Proc. Natl. Acad. Sci. USA 84:9238 (1987); and Bevilacqua et al., Science 243:1160 (1989)); the anti-CD29 (VLA-β chain, 4B4, IgG₁) (Nojima et al., J. Exp. Med. 172:1185 (1990)) and anti-CD18 (H52, IgG₁) (Hildreth et al., J. Immunol. 134:3272 (1985)) mAb were used as diluted ascites fluid (1:100). Anti-HLA class I mAb (W6/32, IgG₂) was used as purified F(ab')₂ fragments (25 µg/ml). The anti-VCAM-1 mAb HAE-2 (Human Activated Endothelium-2; IgG₁) was generated by the fusion of Ag8.653 myeloma cells with spleen cells from BALB/c mice that were repeatedly immunized with HUVEC stimulated for 6 hours with 100 U/ml TNF-α. The specificity of the HAE-2 mAb was determined by its ability to bind recombinant VCAM-1 in a radioimmunoassay (W. Newman, Otsuka America Pharmaceuticals, Inc., Rockville, MD) (Polte et al., Nucl. Acids Res. 18:5901 (1990)) and its immunoprecipitation of a surface iodinated molecule of 110 M_r from activated HUVEC. HAE-2 was used as diluted ascites fluid (1:100). Phosphomannan monoester core complex (PPME) from the yeast *Hansenula holstii* cell wall (provided by Dr. M. Slodki, USDA, Peoria, IL) was used at a final concentration of 3 µg/ml. Heparin (Sigma) was used as a control anionic polysaccharide at a final concentration of 3 µg/ml.

Activation of HUVEC. Confluent endothelial monolayers were stimulated for 2 to 24 hours with recombinant human (rh) TNF-α (100 U/ml; a gift from Asahi, Inc., Japan), rh IL-1β (10 U/ml; a gift from Biogen, Inc., Cambridge, MA), LPS (1 µg/ml; *Escherichia coli* serotype 055:B5; Sigma), rh γ-IFN

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(1000 U/ml; Genzyme, Cambridge, MA) or IL-4 (100 ng/ml; a gift from Immunex Corp., Seattle, WA). Cytokines were in M199 medium containing 10% FCS, heparin and endothelial growth factor. In experiments using cycloheximide (Sigma), HUVEC were preincubated for 30 min. with M199 medium containing 10 µg/ml cycloheximide and stimulated in medium containing 10 µg/ml cycloheximide.

Endothelial-leukocyte attachment assay. An *in vitro* adhesion assay was developed that is similar to the Stamper and Woodruff frozen section assay (Stamper, et al., J. Exp. Med. 144:828 (1976)) as described (Tedder, et al., J. Immunol. 144:532 (1990)), with some modifications. Endothelial cells were grown to confluence on gelatin (0.1%) coated glass slides, stimulated with TNF-α or other inflammatory agents, washed, and incubated for 15 min. with medium, W6/32, HAE-2 or H18/7 mAb, and washed 4 more times. The mAb reactive with endothelial surface molecules were also added back to the leukocyte suspensions so that the mAb were present throughout the assay. Neutrophils (5×10^6) or lymphocytes (4×10^6) were suspended in 100 µl RPMI medium containing 5% FCS and mAb(s) or PPME and incubated for 15 min. on ice prior to addition to the endothelial monolayers with rotation at 64 rpm. After 30 min. incubation the medium was tipped off and the slides were placed vertically in fixative (PBS, 1% (v/v) glutaraldehyde, pH 7.4; Polysciences, Warrington, PA) (Tedder et al., J. Immunol. 144:532 (1990)). After overnight fixation, the endothelial cells were stained with hematoxylin and representative fields were photographed (400X, final magnification). The number of adherent leukocytes was determined by counting 4 to 12 microscopic fields (0.09 mm²/field), and the results were expressed as means ± SD. Fields for analysis were located at half radius distances from the center of the endothelial cultures. All endothelial cell monolayers used in these experiments were confluent at the initiation of the assays. Leukocyte binding to the endothelium and mAb inhibition was

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uniform except at the center of rotation and at the edge of the endothelial monolayers where some endothelial cell detachment occurred. Treatment of endothelial monolayers with PPME (3 μ g/ml) or any of the mAb used in these studies did not alter the integrity of viable HUVEC monolayers. Overnight fixation of confluent endothelial monolayers routinely caused some retraction of the endothelial cells such that the monolayers did not always appear confluent following treatment. However, this slight retraction of the endothelial cells did not alter the number of leukocytes bound per unit area to a detectable extent when compared with unfixed HUVEC monolayers. In addition, carrying out the assays at 4, 27 or 37°C did not affect the integrity of the endothelial cell monolayers.

In additional experiments, TNF-stimulated (6 hours) endothelial cells were treated with neuraminidase (*Vibrio cholerae*, Calbiochem Corp., San Diego, CA) before the addition of leukocytes. Confluent monolayers of HUVEC on glass slides were fixed with 2% glutaraldehyde for 30 min. at 4°C and rehydrated in PBS. Although treatment of the monolayers with glutaraldehyde before the assays reduced leukocyte binding by ~4 fold, this step was necessary as neuraminidase treatment of unfixed HUVEC at pH 5.5 greatly reduced the integrity of the monolayers. The endothelial cells were then overlaid with 100 μ l of neuraminidase (1 U/ml in 50 mM NaOAc, 154 mM NaCl pH 5.5, 4 mM CaCl_2) for 30 min. at 37°C. The slides were washed 5 times in RPMI 1640 medium containing 1% FCS and the assays carried out as above at 4°C. Eight fields were examined and counted on each slide.

Assay for leukocyte attachment to high endothelial venules (HEV) of peripheral lymph nodes. The HEV-binding assay was performed with frozen tissue sections of rat peripheral lymph nodes using the methods of Stamper and Woodruff (Stamper et al., J. Exp. Med. 144:828 (1976)) and Butcher et al. (Butcher et al., J. Immunol. 134:2989 (1979)) as described (Tedder et al., J. Immunol. 144:532 (1990)).

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Briefly, lymphocytes (5×10^6) were treated with neuraminidase (0.005 U/ml) for 30 min. at room temperature, then incubated for 10 min. on ice with the anti-LAM-1 mAbs in medium containing 5% FCS. The cells, in a final volume of 100 μ l, were then incubated under rotation (64 rpm) for 30 min. at 4°C on four 12 μ m frozen rat peripheral lymph node sections. After fixation overnight in PBS with 1% (w/v) glutaraldehyde, the number of HEVs per tissue section was determined and lymphocyte adhesion to HEV was quantitated.

Neutrophil transmigration assay. Neutrophil migration through endothelial cell monolayers was examined using an *in vitro* videomicroscopic transmigration model system that has been described in detail (Luscinskas et al., J. Immunol. 146:1617 (1989)). Briefly, neutrophils (1 ml , 5×10^6) pretreated for 10 min. at 4°C with medium or medium containing mAb were placed on confluent untreated or IL-1 β (10 U/ml) stimulated endothelial monolayers cultured on thick, optically-clear collagen gels that were washed 3 times with RPMI 1640 medium containing 1% FCS. After 20 min. at 37°C, 5% CO $_2$, neutrophil migration across control or IL-1 treated monolayers was assessed using a videomicroscopic system. The data are the percentage of neutrophils that have transmigrated at 20 min.

Statistical analysis. Statistical significance of results was determined using the paired Student's t test.

RESULTS

LAM-1 mediates leukocyte binding to activated endothelium. Anti-LAM-1 mAb were tested for their ability to block lymphocyte and neutrophil binding to cytokine-activated human umbilical vein endothelial cells, using a newly developed assay analogous to the Stamper-Woodruff assay used to assess lymphocyte adhesion to high endothelial venules (HEV) (Stamper et al., J. Exp. Med. 144:828 (1976)). Endothelial cells were grown to confluence on gelatin-coated microscope slides, activated with TNF- α , washed, and then

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overlayered with leukocytes suspended in 100 μ l of medium with gentle rotation (64 rpm) of the slides. The assay was carried out at 4°C, where LAM-1 shedding is minimal (Tedder et al., J. Immunol. 144:532 (1990); and Spertini et al., Nature 349:691 (1991)) and CD18-mediated adhesion is inactive (Marlin et al., Cell 68:805 (1991)). Under these conditions, a major proportion of the attachment of both lymphocytes (48 \pm 13%) and neutrophils (60 \pm 14%) was blocked by the anti-LAM-3 mAb. The treatment of leukocytes and endothelium with mAb reactive with MHC class I antigens and functionally silent epitopes of LAM-1 was without significant effect (~9% inhibition). Thus, neutrophils and lymphocytes both utilize LAM-1 as a major receptor for attachment to activated endothelium.

LAM-1 is involved in the initial attachment of leukocytes to endothelium. The involvement of LAM-1 in leukocyte attachment to endothelium was examined with and without rotation of the slides. When the assays were carried out without rotation, anti-LAM1-3 mAb had no significant effect (7 \pm 7% inhibition, N=3) on lymphocyte or neutrophil adhesion. In contrast, when the attachment assays were performed using rotating conditions, a significant proportion (56 \pm 13%) of lymphocyte (p<0.025) and neutrophil adhesion (p<0.005) was blocked. The control mAb (anti-LAM1-10) gave results similar to medium alone with and without rotation. Although fewer lymphocytes were bound under rotating conditions compared with static assays, similar numbers of neutrophils were bound under both conditions. These results show that LAM-1 is involved in the initial attachment phase of leukocyte adhesion to endothelium and that rotation may serve to minimize the relative contributions of adhesion molecules which may function in adhesive events subsequent to initial attachment. This is in contrast to results obtained using static adhesion assays at 4°C where most lymphocyte attachment was mediated through CD29 and

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neutrophil attachment was mediated through ELAM-1 (Luscinskas et al., J. Immunol. 146:1617 (1991)).

The same epitopes of LAM-1 mediate lymphocyte attachment to HEV and endothelium. In earlier studies, mAb that

5 identify spatially and functionally distinct epitopes on LAM-1 (Spertini et al., J. Immunol. (in press) (1991); and Kansas et al., J. Cell Biol. 114:351 (1991)). The role of these epitopes in ligand binding was examined in lymphocyte attachment to endothelium and attachment to rat lymph node
10 HEV. The anti-LAM1-3, -4 and -6 mAb inhibited ~90% of lymphocyte binding to HEV (Spertini et al., Leukemia 5:300 (1991); and Spertini et al., Nature 349:691 (1991)) and inhibited 36-45% of lymphocyte attachment to endothelium. The anti-LAM1-1 and -7 mAb which respectively inhibit 75 and
15 29% of lymphocyte adhesion to HEV also significantly inhibited lymphocyte adhesion to activated endothelial monolayers. In contrast, four anti-LAM-1 mAb that do not inhibit HEV-binding, or anti-HLA class I mAb, did not inhibit lymphocyte attachment to endothelium. Thus, identical
20 epitopes of LAM-1 mediate binding to endothelium and HEV.

LAM-1 functions in combination with other adhesion receptors to mediate leukocyte attachment to endothelium.

The contribution of LAM-1 mediated attachment to leukocyte-endothelial adhesion was compared with that of other adhesion
25 proteins at 4, 25 and 37°C. At 4°C, mAb reactive with VCAM-1 on endothelium or the β chain of its receptor, CD29, on lymphocytes were able to inhibit lymphocyte adhesion by $60 \pm 8\%$. The combination of anti-LAM1-3 and anti-CD29 mAb inhibited ~90% of lymphocyte adhesion. Neutrophil adhesion
30 was inhibited by mAb binding to endothelial ELAM-1 ($55 \pm 5\%$), and anti-LAM1-3 mAb in combination with anti-ELAM-1 mAb blocked ~90% of binding. The anti-CD29 mAb had no detectable effect on neutrophil binding to endothelium and the anti-ELAM-1 mAb had no significant effect on lymphocyte binding
35 to endothelium, further demonstrating the specificity of inhibition. CD18 played a minor role in lymphocyte and

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neutrophil adhesion at 4°C. Treatment of cells with mAb reactive with functionally silent epitopes of LAM-1 or with anti-class I mAb did not inhibit leukocyte adhesion.

5 MAb reactive with CD18 inhibited lymphocyte (30%) and neutrophil (39%) adhesion to endothelium at 25°C. Similarly, anti-LAM1-3 mAb inhibited adhesion by 47% for lymphocytes and 46% for neutrophils. The combination of anti-LAM1-3 and anti-CD29 mAb inhibited a major portion of lymphocyte attachment (64%), while the combination of these two mAb with
10 anti-CD18 mAb inhibited 88 to 90% of adhesion. The combination of anti-LAM-1 and anti-ELAM-1 mAb inhibited a significant portion of neutrophil attachment (44%), and the combination of these two mAb with anti-CD18 mAb also inhibited 88 to 90%.

15 At 37°C, lymphocyte adhesion was significantly inhibited (56%) by anti-LAM1-3 alone ($p < 0.05$), while anti-CD18 mAb inhibited by 43%, and anti-CD29 mAb inhibited by 65%. The combination of these mAb inhibited the binding of lymphocytes by ~90%. Neutrophil adhesion was inhibited by anti-LAM-1-3
20 (27%), CD18 (22%) and anti-ELAM-1 (26%) mAb. Again, combining the anti-LAM-1, anti-ELAM-1 and CD18 mAb blocked ~90% of adhesion. Thus, LAM-1 acts in concert with other adhesion molecules to mediate leukocyte attachment to cytokine activated endothelium, and in this assay system,
25 these receptors in combination can account for nearly all leukocyte attachment.

Role of LAM-1 in leukocyte transmigration. Leukocyte entry into sites of inflammation *in vivo* is likely to involve multiple receptor systems. Therefore, the role of LAM-1 in
30 leukocyte extravasation was examined using an *in vitro* videomicroscopic transmigration model system (Luscinskas et al., J. Immunol. 146:1617 (1991)). In this assay, treatment of neutrophils with mAb against HEV-binding epitopes of LAM-1 significantly inhibited transmigration (~30-43%, $p < 0.001$),
35 in comparison to treatment with mAb reactive with CD16, functionally silent epitopes of LAM-1, or HLA class I

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antigens which were without significant effect. The increase in migration induced by cytokine activation of endothelial monolayers was quantitatively blocked by anti-CD18 or anti-ELAM-1 mAb. Thus, in this functional assay, LAM-1 contributes, in conjunction with other adhesion receptors, to the enhanced neutrophil transmigration observed following cytokine activation of endothelial monolayers.

Biochemical properties of the endothelial ligand. PPME is a soluble carbohydrate which specifically inhibits lymphocyte binding to HEV (Yednock et al., J. Cell Biol. 104:713 (1987); Yednock et al., J. Cell Biol. 104:725 (1987); and Stoolman et al., Blood 70:1842 (1987)). Therefore, it was examined for its ability to inhibit leukocyte interactions with endothelium. PPME significantly inhibited (p<0.001) lymphocyte and neutrophil attachment to activated endothelium to the same extent as the anti-LAM1-3 mAb. This effect was specific for PPME as another anionic polysaccharide, heparin at 3 μ g/ml, did not inhibit lymphocyte or neutrophil attachment to activated endothelium as similarly described for HEV binding (Yednock et al., J. Cell Biol. 104:725 (1987)).

The ligand for LAM-1 on HEV is composed, in part, of sialic acid as neuraminidase treatment of HEV eliminates LAM-1 mediated attachment (Rosen et al., Science 228:1005 (1985); and True et al., J. Cell. Biol. 111:2757 (1990)). Therefore, the role of sialic acid in LAM-1 mediated attachment to activated endothelium was also examined. Fixed endothelial cells bound 8 ± 4 neutrophils per unit area and 4 ± 2 after neuraminidase treatment. TNF- α treatment of the endothelial cells for 6 hours before fixation increased leukocyte binding to 461 ± 117 neutrophils. Treatment of the endothelial cells with neuraminidase reduced binding by 63% to 168 ± 52 neutrophils while treatment of the neutrophils with the LAM1-3 mAb decreased binding by 78% to 102 ± 28 . Treatment of endothelium with neuraminidase in conjunction with treatment of neutrophils with anti-LAM1-3 mAb did not increase the

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levels of inhibition (66%), resulting in 157 ± 34 neutrophils binding. Therefore, as with HEV, the LAM-1 ligand expressed by activated endothelium appears to contain carbohydrate residues bearing sialic acid as an essential component.

5 Inflammatory cytokines induce expression of the LAM-1
 ligand by endothelial cells. Leukocyte attachment to
unactivated endothelium at 4°C was minimal, with less than
10 leukocytes bound per field. Stimulation of endothelium
10 with TNF- α induced a dramatic increase in both lymphocyte and
neutrophil adhesion to endothelium. IL-1 β or bacterial
endotoxin (LPS) also resulted in dramatically increased
lymphocyte adhesion, while treatment with γ -IFN or IL-4
induced smaller, but significant ($p < 0.005$), increases. In
15 all cases, treatment of lymphocytes or neutrophils with anti-
LAM1-3 mAb blocked attachment by 50 to 70%. It is unlikely
that cytokine treatment affected leukocyte function since the
endothelium was washed extensively prior to leukocyte
binding. Therefore, different inflammatory cytokines and
20 agents were able to induce different levels of leukocyte
adhesion mediated through LAM-1.

Kinetics of endothelial cell expression of the LAM-1
 ligand. Untreated endothelium or endothelium cultured with
TNF- α for two hours supported little lymphocyte binding at
4°C. However, a significant increase in lymphocyte adhesion
25 was observed after 4 to 24 hours of TNF- α stimulation. Anti-
LAM1-3 specifically inhibited the increased attachment by 36-
69%, while mAb against HLA class I antigens or nonfunctional
epitopes of LAM-1 were without effect. A similar degree of
inhibition of binding was obtained with the anti-CD29 mAb,
30 and the combination of anti-LAM1 and anti-CD29 mAb inhibited
most lymphocyte adhesion. Induction of adhesive ligands was
dependent on protein synthesis since cyclohexamide (10 μ g/ml)
treatment of endothelium completely abrogated adhesion.
Therefore, expression of the LAM-1 ligand is induced between
35 2 and 4 hours following endothelial stimulation, and persists
for at least 24 hours.

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The ability of neutrophils to bind to TNF-stimulated endothelium during this time period was also examined. Although endothelial cells stimulated for 6 hours bound high numbers of neutrophils (800-1000 cells/field), cells stimulated for 24 hours bound only 114 ± 12 neutrophils at 4°C . Stimulated endothelium treated with anti-HLA-class I mAb bound 127 ± 17 neutrophils per field. Treatment of the neutrophils with anti-LAM1-3 mAb reduced binding by 82% (20 ± 5 cells bound) while treatment of the endothelial cells with anti-ELAM-1 mAb blocked binding by 65% (40 ± 11 cells bound). Treatment of the cells with anti-LAM1-3 and anti-ELAM-1 mAb together inhibited neutrophil binding by 95% (5 ± 3). After 36 hours of endothelial cell stimulation, anti-LAM1-3 mAb blocked 75% of neutrophil binding (39 ± 23 cells/field), the anti-ELAM-1 mAb blocked only 21% of binding (118 ± 45), while the anti-LAM1-3 and anti-ELAM-1 mAb together blocked 75% of binding (37 ± 14), in comparison with the anti-LAM1-10 mAb that did not inhibit binding (150 ± 50). These results suggest that at later time points when endothelial cell expression of ELAM-1 and neutrophil adhesion is diminished, LAM-1 contributes relatively more to the interactions of activated endothelium with neutrophils.

Use

Leukocyte migration and infiltration into areas of tissue damage or injury or tissue transplant in a patient can cause or increase pathology. Therefore, methods for detecting sites of damage and agents that impede these infiltration processes can be used for therapeutic treatment. Specifically, leukocyte-mediated inflammation is involved in a number of human clinical manifestations, including the adult respiratory distress syndrome, multi-organ failure and reperfusion injury. One way of inhibiting this type of inflammatory response is to image the site of inflammation and to block competitively the adhesive interactions between leukocytes and the endothelium adjacent to the inflamed

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region. On a systemic level, treatment of a patient in shock (e.g., from a serious injury) with an antagonist to cell surface LAM-1 function can result in the reduction of leukocyte migration and adhesion to a level manageable by the target endothelial cells and the subsequent dramatic recovery of the patient.

In addition, subpopulations of malignant cells that express the LAM-1 receptor protein would allow the receptor to function in metastasis of tumor cells. Agents developed to block receptor function can inhibit the metastasis and homing of the malignant cells.

One method of imaging the sites of inflammation in a patient involves detecting the expression of the LAM-1 ligand on the inflamed endothelium. The method includes administering to a patient a pharmaceutical composition consisting of a detectable amount of a labeled LAM-1 protein, or ligand binding fragment of a LAM-1 protein, alone or joined to a carrier protein in a pharmaceutically acceptable carrier. Sufficient time is allowed for the labeled protein to localize at the site of LAM-1 ligand expression, unbound protein is permitted to clear from healthy tissue in the patient, and signal generated by the label is detected and converted into an image of the site of inflammation. The amount labeled LAM-1 protein preferably would be from 1 pg/kg to 10 mg/kg although higher or lower doses are possible depending on the imaging agent label used and the sensitivity of the detection method.

Some of the labels which can be detected externally from within the body of a human patient include radionuclides, radiopaque labels, and paramagnetic isotopes. A radionuclide for in vivo diagnosis should have a half-life long enough that it is still detectable at the time of maximum uptake, but short enough that after diagnosis unwanted radiation does not remain in the patient. Coupling of radionuclides to antibodies or proteins is well known in the art (see, e.g., Daddona et al., U.S. Pat. No. 5,026,537, the teachings of

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which are incorporated by reference herein) and is often accomplished either directly or indirectly using an intermediary functional group such as a chelating agent. Examples of radioisotopes that could be used for *in vivo* diagnosis are ^{99}Tc , ^{123}I , ^{131}I , ^{111}In , ^{97}Ru , ^{97}Cu , ^{67}Ga , ^{68}Ga , ^{72}As , ^{89}Zr , and ^{201}Tl . Paramagnetic isotopes for purposes of *in vivo* diagnosis can also be used according to the methods of this invention. Examples of elements that are particularly useful for use in magnetic resonance energy techniques include ^{157}Gd , ^{55}Mn , ^{162}Dy , ^{52}Cr , and ^{56}Fe . For radiopaque imaging, the LAM-1 protein or ligand binding fragment is coupled to an agent that produces an opaque field at the site of inflammation upon X-ray imaging.

Individual domains of LAM-1, ligand binding fragments of LAM-1, or the entire LAM-1 protein can then be used for therapeutic treatment to interfere in the binding of leukocytes (expressing the LAM-1 protein) at the site of inflammation. In addition, the LAM-1 protein, a specific domain, or a ligand binding fragment can be joined to a carrier protein to increase the serum half-life of the therapeutic agent. For example, a LAM-1 fusion protein with human IgG1 heavy chain, an antibody-like immunoglobulin chimera, was produced as follows: an altered fragment of LAM-1 cDNA was produced that generated a *Ban* II endonuclease cleavage site within the exon VIII encoded domain by PCR using an antisense primer with the sequence GTTATAATCGGGCTCCTTAATC. This generated a *Ban* II sequence at nucleotide positions 1073-1078 (Fig. 2). The LAM-1 cDNA fragment encoding the leader, lectin, EGF-like and SCR domains plus this altered spacer domain were fused at this *Ban* II site to the *Ban* II site within the hinge region of a human IgG1 heavy chain cDNA. This generated a cDNA encoding the LAM-1 extracellular domains fused with the hinge, CH2, and CH3 domains of IgG1. This cDNA was subcloned into a mammalian expression vector and was subsequently transfected into COS-7 cells. The soluble dimeric LAM-1/IgG1 protein

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product secreted into the medium retained all of the antigenic epitopes of the LAM-1 molecule defined by 16 anti-LAM-1 monoclonal antibodies. In a similar fashion a soluble immunoglobulin chimera can be obtained for each specific exon-encoded domain of LAM-1, or fragment thereof. The immunoglobulin chimera are easily purified through IgG-binding protein A-Sepharose chromatography. The chimera have the ability to form an immunoglobulin-like dimer with the concomitant higher avidity and serum half-life.

Other agents can also be joined to the LAM-1 protein or to a specific domain or ligand binding fragment to form a useful product. For example, the LAM-1 lectin domain can be combined with the toxic portion of a cytotoxin to produce a fusion protein. In addition, the LAM-1 protein or protein portions may be coupled to a chemotherapeutic drug or drugs which could bind to cells expressing the LAM-1 ligand, to administer the drug to a site of tissue damage or inflammation to treat, e.g., acute inflammation or vasculitis. Such drugs may include, anti-inflammatory agents or agents that provide regional relief from inflammatory distress. Syndromes, diseases, and conditions that could be treated by these agents would include, but not be limited to, treating inflammation, microbial/parasitic infections, post-reperfusion injury, leukemia, lymphoma, vasculitis, inhibition of the metastatic spread of tumor cells, organ transplantation, or graft rejection. The fusion proteins can be transcribed from a cDNA hybrid molecule, as described above, or the agent may be covalently bonded to the LAM-1 protein or domain by routine procedures.

A ligand binding fragment of the LAM-1 protein can be experimentally determined using this invention. In a typical procedure, fragments of the LAM-1 cDNA can be fused with cDNA encoding a carrier protein, such as immunoglobulin heavy chain on the CD62 cDNA as described by Kansas et al. (J. Cell Biol. 114:351 (1991) hereby incorporated by reference herein). These chimeric proteins can be expressed on the

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cell surface, or as soluble molecules, and their ability to bind ligand assessed. For example, the ability of LAM-1 fusion proteins to bind PPME or fucoidin requires only the lectin domain of LAM-1 (Kansas et al., supra). Further segregation of fragments of the LAM-1 protein or peptides from the LAM-1 protein can be examined for their ability to bind ligands, as well as their ability to inhibit the binding of LAM-1 protein to ligand.

A method for detecting LAM-1 ligand expression in a biological sample uses the LAM-1/ligand binding reaction and comprises contacting the sample with the LAM-1 protein or LAM-1⁺ cells, and detecting whether a complex forms. This invention allows the detection and biochemical identification of the LAM-1 ligand. First, the ligand can be identified directly by the production of monoclonal antibodies reactive with molecules expressed on activated endothelium that are able to inhibit the attachment of leukocytes to the endothelial cells through LAM-1. These blocking antibodies can be produced and tested by routine procedures and used to identify the endothelial ligand and its tissue distribution and to isolate and purify the ligand for biochemical analysis and amino acid sequence determination. Clones of cDNA and genomic DNA encoding the LAM-1 ligand can also be isolated by standard procedures and used to synthesize the ligand for therapeutic utility.

In addition, the use of this assay will allow the development of antagonists which can inhibit the function of LAM-1. We have already developed one class of antagonists composed of monoclonal antibodies that bind to ligand-binding epitopes on LAM-1 and thus inhibit LAM-1 function. Alternatively, LAM-1 fragments or ligand binding-domains fused with other proteins can be tested for their ability to inhibit the binding of leukocytes to endothelium through LAM-1. For example, antagonists have been developed which are composed of the LAM-1 extracellular domains fused with the hinge, CD2 and CD3 domains of immunoglobulin heavy chain.

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These agents inhibit LAM-1 mediated attachment of leukocytes. Another similar construct composed of LAM-1 fragments that has been examined using this invention is a fusion protein composed of the ligand binding domain of LAM-1 fused with the EGF-like and short consensus repeat domains of CD62 bound to a cell surface. In a similar fashion the ligand binding domain of LAM-1 can be substituted for the ligand binding domain of another selection such as ELAM-1 or GMP-140 to form an antagonistic construct. This invention will allow the development of pharmacologic reagents composed of peptides, carbohydrate moieties, RNAs or other small molecules which may mimic the LAM-1 ligand or ligand-binding epitopes of LAM-1, and inhibit the attachment of leukocytes to endothelium through this receptor.

In another therapeutic method the LAM-1 protein or ligand binding fragment, or the LAM-1 ligand or fragment thereof can be used in combination therapy with any other selectin or any other cell surface molecule, or soluble fragments thereof, involved in adhesion of leukocytes to endothelial surfaces. In addition, antagonists to any of the above receptors or receptor portions (or mAb reactive to the receptors or receptor portions) can be used in the above combinations or as independent antagonist combinations for treatment of a patient. Examples include ICAM-1, VCAM-1, VLA-4, CD18, CD11a, CD11b, CD31, GMP-140, and ELAM-1 and the mAb reactive with them.

In addition, other tissues can be examined for the presence of the endothelial LAM-1 ligand. For instance, following reperfusion injury, vascular endothelium in multiple organs may express the LAM-1 ligand. Furthermore, soluble (non-cell bound) LAM-1 ligand-binding agents that are labeled directly or detected by antibodies reactive with the LAM-1 fragment can be used to detect and quantify LAM-1 ligand in biological fluids, on a cell surface, or bound to a solid matrix such as nitrocellulose. For example, a tissue biopsy sample can be reacted with LAM-1 or a ligand-binding

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fragment thereof, and the binding of LAM-1 monitored using standard immunological or immunohistological assays. The reactivity of LAM-1 with this tissue would be a measure of inflammation and thereby a prognostic indicator, such as in the case of transplanted organ rejection. Alternately, the LAM-1 ligand can be isolated from endothelium by forming a LAM-1 protein/ligand complex in a sample, which may then be separated from a reaction mixture by contacting the complex with an immobilized antibody or protein which is specific for LAM-1 or a carrier protein containing a ligand binding fragment of LAM-1. The isolated LAM-1 ligand can be biochemically identified and purified by standard techniques or the LAM-1/ligand complex can be quantitated to give a measure of endogenous LAM-1 ligand and, thereby, the extent of stimulation of endothelial cells. Of course, the specific concentrations of detectably labeled LAM-1 or ligand-binding fragment thereof and the temperature and time of incubations, as well as other assay conditions, may be varied depending on various factors including the concentration of LAM-1 ligand in the sample. These parameters can be determined by those skilled in the art.

Moreover, the LAM-1 ligand may be shed from the cell surface following activation of the endothelium during various inflammatory reactions. The LAM-1 ligand assay or other standard immunological assays (e.g., an ELISA assay) using LAM-1, or ligand-binding fragments thereof, may be used to assess the extent of LAM-1 ligand shedding and to permit monitoring of the progress of an inflammatory event, microbial infection, vasculitis or organ transplant rejection by the presence of a LAM-1 binding component of the ligand in body fluids. Typical fluids that can be monitored are serum, plasma, cerebrospinal fluid, urine, saliva, semen, lymph or other secretions.

The therapeutic agents may be administered orally, topically, or parenterally, (e.g., intranasally, subcutaneously, intramuscularly, intravenously, or intra-

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arterially) by routine methods in pharmaceutically acceptable inert carrier substances. Optimal dosage and modes of administration can readily be determined by conventional protocols.

5 The *lyam-1* gene itself can also be used in genetic therapy. Individuals having a genetic defect in the *lyam-1* gene would be unable to produce a fully active LAM-1 protein leukocyte "homing" receptor and thus would be unable to mobilize sufficient leukocytes to a site of inflammation.
10 Individuals suspected to having a congenital defect in the *lyam-1* gene could be screened for this genetic disorder using the sequence and structural information described. Treatment of affected individuals would then be possible using the *lyam-1* gene or fragments thereof.

15 The normal regulation of the *lyam-1* gene, as evidenced by the appearance and disappearance of the LAM-1 protein on the surface of a specific leukocyte sub-population can be monitored to test the effects of drugs or specific therapies that would alter gene expression.

20 Other embodiments are within the following claims.

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CLAIMS

What is claimed is:

1. A method of detecting a site of inflammation or a diseased state in a human patient comprising administering to said patient a pharmaceutical composition comprising a detectable amount of a labeled LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance, and detecting the label on said LAM-1 protein.
2. The method of claim 1 wherein said LAM-1 protein or ligand binding fragment thereof is labeled with any one of a radionuclide, a paramagnetic isotope, or a radiopaque label.
3. The method of claim 1 wherein said LAM-1 protein or ligand binding fragment thereof is joined to a carrier protein.
4. The method of claim 3 wherein said carrier protein comprises an immunoglobulin heavy chain constant region.
5. The method of claim 3 wherein said carrier protein comprises non-ligand binding portions of a selectin molecule.
6. An imaging agent for imaging a site of inflammation or a diseased state in a human patient comprising LAM-1 protein or a ligand binding fragment thereof labeled with a detectable label and suspended in a pharmaceutically acceptable carrier substance.
7. The imaging agent of claim 6 wherein said detectable label comprises any one of a radionuclide, a paramagnetic isotope, or a radiopaque label.

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8. The imaging agent of claim 6 wherein said LAM-1 protein or ligand binding fragment thereof is joined to a carrier protein.

9. The imaging agent of claim 9 wherein said carrier protein comprises an immunoglobulin heavy chain constant region.

10. The imaging agent of claim 9 wherein said carrier protein comprises non-ligand binding portions of a selectin molecule.

11. A method of treating a human patient suffering from a leukocyte-mobilizing condition comprising administering to said patient a therapeutic composition comprising a therapeutic amount of LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance.

12. The method of claim 11 wherein said patient suffers from tissue damage or an autoimmune disorder.

13. The method of claim 11 wherein said patient suffers from cancer.

14. The method of claim 11 wherein said patient is an organ or tissue transplant recipient.

15. The method of claim 11 wherein said LAM-1 protein or ligand binding fragment thereof is joined to a therapeutic agent.

16. The method of claim 15 wherein said therapeutic agent comprises either a chemotherapeutic drug or an anti-inflammatory agent.

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17. A method of treating a human patient suffering from a leukocyte-mobilizing condition comprising administering to said patient a therapeutic composition comprising a therapeutic amount of an antagonist to LAM-1 protein or a ligand binding fragment thereof in a pharmaceutically acceptable carrier substance.
18. The method of claim 17 wherein said patient suffers from tissue damage or an autoimmune disorder.
19. The method of claim 17 wherein said patient suffers from cancer.
20. The method of claim 17 wherein said patient is an organ or tissue transplant recipient.
21. The use of LAM-1 protein or ligand binding fragment thereof to identify a ligand that binds to said protein.
22. The use of LAM-1 protein or ligand binding fragment thereof to identify a ligand that binds to a molecule specifically associated with said protein, or a fragment thereof.
23. The method of claim 17 wherein said antagonist comprises a ligand to LAM-1 protein, or a portion thereof.
24. The method of claim 11 or claim 17 further comprising administering to said patient a therapeutic amount of a cell surface molecule other than LAM-1 protein or ligand binding fragment thereof, or soluble fragment of said cell surface molecule, said cell surface molecules or soluble fragments being capable of participating in the adhesion of leukocytes to endothelial surfaces.

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25. The method of claim 11 or claim 17 further comprising administering to said patient a therapeutic amount of an antagonist to a cell surface molecule other than LAM-1 protein or ligand binding fragment thereof, or soluble fragment of said cell surface molecule, said cell surface molecules or soluble fragments being capable of participating in the adhesion of leukocytes to endothelial surfaces.

26. A method of determining the extent of binding of blood leukocytes to activated human endothelium comprising the steps of

- providing a monolayer of human endothelial cells grown to confluence on a solid support,
- activating said confluent endothelial cells,
- rotating the endothelial cells on the solid support in said suspension of leukocytes,
- removing said suspension, and
- determining the number of leukocytes adhering to said endothelial cells.

FIG. 1A

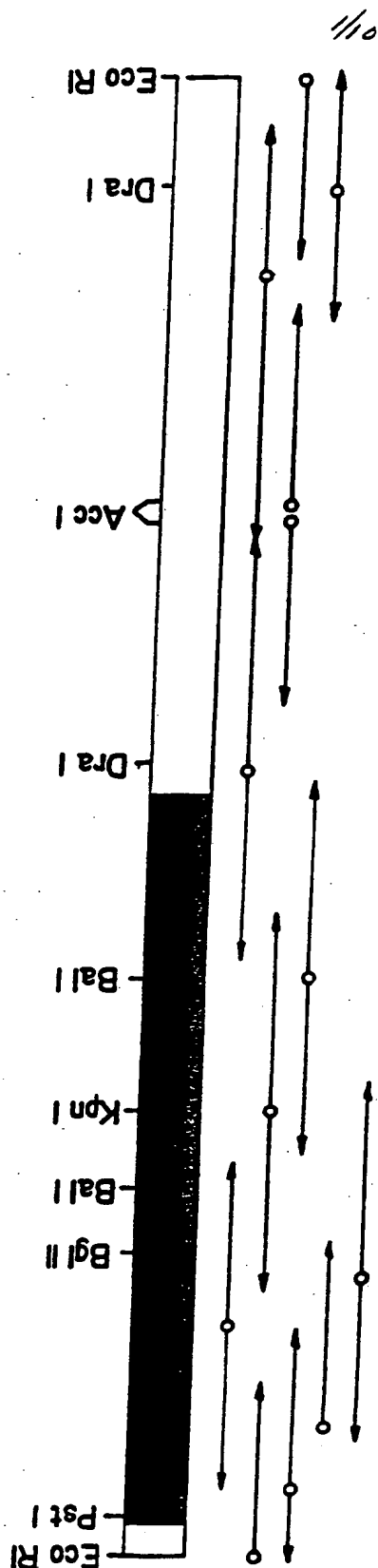


FIG. 1B



2/10

1 M G
 8aattcCCTTT GGGCAAGGAC CTGAGACCCT TGTGCTAAGTCAAGAGGCTCA ATG GGC
 10
 C R R T R E G P S K A M
 TGC AGA AGA ACT AGA GAA GCA CCA AGC AAA GCC ATG 94
 20
 I F P W K C Q S T Q R D L W N I
 ATA TTT CCA TGG AAA TGT CAG AGC ACC CAG AGG GAC TTA TGG AAC ATC 30
 40
 F K L W G W T M L C C D
 TTC AAG TTG TGG GGG TGG ACA ATG CTC TGT TGT GAT 168
 50
 F L A H H G T D C W T Y H Y S E
 TTC CTG GCA CAT CAT GGA ACC GAC TGC TGG ACT TAC CAT TAT TCT GAA 60
 K P M N W Q R A R R F C
 AAA CCC ATG AAC TGG CAA AGG GCT AGA AGA TTC TGC 262
 80
 R D N Y T D L V A I Q N K A E I
 CGA GAC AAT TAC ACA GAT TTA GTT GCC ATA CAA AAC AAG GCG GAA ATT 90
 E Y L E K T L P F S R S
 GAG TAT CTG GAG AAG ACT CTG CCT TTC AGT CGT TCT 346

FIG. 2A

SUBSTITUTE SHEET

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100 Y Y W I G I R K I G G 110 W T W
 TAC TAC TGG ATA GGA ATC CGG AAG ATA GGA GGA ATA TGG ACG TGG
 120 V G T N K S L T E E A E N
 GTG GGA ACC AAC AAA TCT CTC ACT GAA GAA GCA GAG AAC 430
 130 W G D G E P N N K K N K E D C
 TGG GGA GAT GGT GAG CCC AAC AAC AAG AAG AAC AAG GAG GAC TGC
 150 V E I Y I K R N K D A G K
 GTG GAG ATC TAT ATC AAG AGA AAC AAA GAT GCA GGC AAA 514
 160 W N D D A C H K L K A A L C Y
 TGG AAC GAT GAC GCC TGC CAC AAA CTA AAG GCA GCC CTC TGT TAC
 170 T A S C Q P W S C S H G
 ACA GCT TCT TGC CAG CCC TGG TCA TGC AGT GGC CAT GGA 598
 180 E C V E I I N N Y T C N C D V
 GAA TGT GTA GAA ATC ATC AAT AAT TAC ACC TGC AAC TGT GAT GTG
 200 G Y Y G P Q C Q F V I Q C
 GGG TAC TAT GGG CCC CAG TGT CAG TTT GTG ATT CAG TGT 682
 220 E P L E A P E L G T M D C T H
 GAG CCT TTG GAG GCC CCA GAG CTG GGT ACC ATG GAC TGT ACT CAC
 230 P L G N F N F N S Q C A F
 CCT TTG GGA AAC TTC AAC TTC AAC TCA CAG TGT GCC TTC 766
 240 S C S E G T N L T G I E E T T
 AGC TGC TCT GAA GGA ACA AAC TTA ACT GGG ATT GAA GAA ACC ACC
 260 C E P F G N W S S P E P T
 TGT GAA CCA TTT GGA AAC TGG TCA TCT CCA GAA CCA ACC 850
 270 C Q V I Q C E P L S A P D L G
 TGT CAA GTG ATT CAG TGT GAG CCT CTA TCA GCA CCA GAT TTG GGG
 290 I M N C S H P L A S F S F
 ATC ATG AAC TGT AGC CAT CCC CTG GCC AGC TTC AGC TTT 934
 300 T S A C T F I C S E G T E L I
 ACC TCT GCA TGT ACC TTC ATC TGC TCA GAA GGA ACT GAG TTA ATT
 310 G K K K T I C E S S G I W
 GGG AAG AAG AAA ACC ATT TGT GAA TCA TCT GGA ATC TGG 1018
 330 S N P S P I C Q K L D K S F S
 TCA AAT CCT AGT CCA ATA TGT CAA AAA TTG GAC AAA AGT TTC TCA
 340 M I K E G D Y N P L F I P
 ATG ATT AAG GAG GGT GAT TAT AAC CCC CTC TTC ATT CCA 1102

FIG. 2B

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V A V M V T A F S G L A F I I
 GTG GCA GTC ATG GTT ACT GCA TTC TCT GGG TTG GCA TTT ATC ATT
 360
 W L A R R L K K G K K S K
 TGG CTG GCA AGG AGA TTA AAA AAA GGC AAG AAA TCC AAG 1186
 370
 R S M N D P Y *
 AGA AGT ATG AAT GAC CCA TAT TAA ATCGCCCTTG GTGAAAGAAA
 380
 ATTCTTGGA TACTAAAAAT CATGAGATCC TTAAATCCT TCCATGAAAC 1280
 GTTTTGTGTG GTGGCACCTC CTACGTCAAA CATGAAGTGT GTTTCCTTCA
 GTGCATCTGG GAAGATTCT ACCTGACCAA GAGTTCCTTC AGCTTCCATT 1380
 TCACCCCTCA TTTATCCCTC AACCCCCAGC CCACAGGTCT TTATACAGCT
 CAGCTTTTTC TCTTTTCTGA GGAGAAACAA ATAACACCAT AAAGGGAAAG 1480
 GATTCATGTG GAATATAAG ATGGCTGACT TTGCTCTTTC TTGACTCTTG
 TTTTCAGTTT CAATTCAGTG CTGTA CTGTA TGACAGACAC TTCTAAATGA 1580
 AGTGCAAATT TGATACATAT GTGAATATGG ACTCAGTTTT CTTCAGATC
 AAATTTGCGG TCGTCTTCTG TATACGTCCA GGTACACTCT ATGAAGTCAA 1680
 AAGTCTACGC TCTCCTTTCT TTCTAACTCC AGTGAAGTAA TGGGGTCTCG
 CTCAAGTTGA AAGAGTCCTA TTTGCACTGT AGCCTCGCCG TCTGTGAATT 1780
 GGACCATCCT ATTTAACTGG CTTCAGCCTC CCCACCTTCT TCAGCCACCT
 CTCCTTTTCA GTTGGCTGAC TTCCACACCT AGCATCTCAT GAGTGCCAAG 1880
 CAAAAGGAGA GAAGAGAGAA ATAGCCTCCG CTGTTTTTTA GTTTGGGGGT
 TTTGCTGTTT CCTTTTATGA GACCCATTCC TATTTCTTAT AGTCAATGTT 1980
 TCTTTTATCA CGATATTATT AGTAAGAAAA CATCACTGAA ATGCTAGCTG
 CAACTGACAT CTCTTTGATG TCATATGGAA GAGTTAAAC AGGTGGAGAA 2080
 ATTCCTTGAT TCACAATGAA ATGCTCTCCT TTCCCCTGCC CCCAGACCTT
 TTATCCACTT ACCTAGATTC TACATATTCT TTAAATTTCA TCTCAGGCCT 2180
 CCCTCAACCC CACCACTTCT TTTATAACTA GTCCTTTACT AATCCAACCC
 ATGATGAGCT CCTCTTCCTG GCTTCTTACT GAAAGGTTAC CCTGTAACAT 2280
 GCAATTTTGC ATTTGAATAA AGCCTGCTTT TTAAGTGTTA AAAgaattc 2330

FIG. 2C

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FIG. 3A

LAM-1 35 GIMLCC-DFLAHHGTDCTWTHYSEKPMNQRRRHCNDYTDVAIQN-
 FcE-R 175 GFVNCVILPEKWINFQRKC--MYPGLGTKQWVHARYACDDMEGQLVSIHS-
 C-HL 75 LFP CGAOSRQWEYFEGRC--MYFSLSNMWHKAKAECEEMHSHIILIDS-
 H-MBP 118 NGIYQKCLTESLGKQVNLFFLTNGE--IMTFELVLALC-VKFPPLWPPPG-
 F-PGC 452 GQDTEICDYGWKFQGC--MYFAHRRIMDAARECR LQGAHITSLLS-
 HHL-1 148 GSERTCCPVNWVEHERSC--WMPERSGKAWADADNYCRLENAHLVAVTS-
 ISL 17 IFISTAAVPQLQKALDGREYLIEITELKYNWQWHEQARHDQQLVITESA

LAM-1 --KAEIYELDKTLPFSRYWIGIRKIG--GIMWV-GINKSLTEEAENW
 FcE-R --PEE--QDFLTILHASTGSMWIGIRNLDLK--GEFIMVDCSHVD--YSNW
 C-HL --YAK--QNFVMFRTRNERFWIGLTDENQE--CEMQWVDGTDTRSS--FTFW
 H-MBP --MAA--EKGAIQNLILEEAFDGMPELTE--GQF--VDIIGNRLT--YTINW
 F-PGC --HEE--QMFVNRVGHDIQ--WIGLNDKMF--HDEFMTDCS--TLOQENW
 HHL-1 --WEE--QKPVQHHIGPVNTMMLHDQN--GPMKWVDGTDYE-TG-PKINW
 ISL DKNNATIDVGRVVVGKSHNLM LGND EYSRDYCPFFMS-PIGQAFS--FAYW

LAM-1 GDGEPNNKKNK--EDCVETIVIKRNKDAKGNDDACH-KIKAAALQYT 160
 FcE-R APGEPTSRSQ--G--EDCVMM--R--GSGRWNDAFCDRKLCAWVCDR 284
 C-HL LE GEPNNR--GFNEDCAH--VTSCQWNDVYCTYECY--VCEL 203
 H-MBP NE GEPNNA--GSDPFCVL--LIKNGQWNDSPCF-HDP SACEF 245
 F-PGC RPNQD SFFSA--G--EDCVMTI--WHENGQWNDVPCNYHLY-TCKK 580
 HHL-1 RPEQPDWYCHGLGGCEDCAH--PTDGRWNDVVCQ-RPYRWVCEI 279
 ISL SENNPNNYHLHQ--EFCHVHWNTLPLY--QWDDDCN-VINGYICEP 159

LAM-1	173	C	Q	P	-	-	-	W	S	C	S	G	H	G	E	C	V	E	I	I	N	-	-	Y	T	C	N	C	D	V	G	Y	Y	G	P	Q	C	Q	205	
EGF	6	C	P	L	S	H	D	G	Y	C	L	H	D	G	V	C	M	Y	I	E	A	L	D	K	Y	A	C	N	C	V	V	G	Y	I	G	E	R	C	Q	43
F-IX	51	C	E	S	-	-	-	N	P	C	L	N	G	G	S	C	K	D	I	N	S	-	-	Y	E	C	W	C	P	F	G	F	E	G	K	N	C	E	83	
F-PGCP	382	C	K	M	-	-	-	N	P	C	K	N	G	G	T	C	Y	P	T	E	T	S	-	-	Y	V	C	T	C	V	P	G	Y	S	G	D	Q	C	E	414

FIG. 3B

LAM-1	207	V	I	Q	C	E	P	L	E	A	P	E	L	G	T	M	D	C	T	H	P	L	G	N	F	N	-	S	Q	C	A	F	-	-		
LAM-1	269	V	I	Q	C	E	P	L	S	A	P	D	L	G	I	M	N	C	S	H	P	L	A	S	F	S	F	T	-	S	A	C	T	F	-	-
Ba	137	A	G	Y	C	S	N	P	G	I	P	-	I	G	T	R	K	V	G	S	Q	Y	R	L	E	D	-	-	-	S	V	-	T	Y	-	-
CR1	694	V	-	-	C	Q	P	P	E	I	L	H	-	G	E	H	T	P	S	H	Q	D	-	N	F	S	-	P	G	Q	E	V	F	Y	-	-
IL-2R	101	P	G	H	C	R	E	P	P	-	P	W	E	N	E	A	T	E	R	I	Y	H	F	V	V	G	-	Q	M	V	Y	Y	Q	-	-	-
F-XIII	1	E	K	P	C	G	F	P	H	V	E	N	G	R	I	A	Q	Y	Y	T	F	K	S	F	Y	F	P	M	S	I	D	K	K	L	S	F

LAM-1	268	S	C	S	E	G	T	N	-	L	-	T	G	I	E	E	-	T	T	C	-	-	-	E	P	F	G	N	W	S	S	P	E	P	T	C	Q
LAM-1	330	I	C	S	E	G	T	E	-	L	-	I	C	K	K	K	-	T	I	C	-	-	-	E	S	S	G	I	W	S	N	P	S	P	I	C	Q
Ba	194	H	C	S	R	G	L	T	-	L	-	R	G	S	Q	R	-	R	T	C	-	-	-	Q	E	G	G	S	W	S	G	T	E	P	S	C	Q
CR1	751	S	C	E	P	G	Y	D	-	L	-	R	G	A	A	S	-	L	H	C	-	-	-	T	P	Q	G	D	W	S	D	E	A	P	R	C	A
IL-2R	164	-	C	V	Q	G	Y	R	A	L	H	R	G	P	A	E	-	S	V	C	K	M	T	H	G	K	T	R	W	T	Q	P	Q	L	I	C	T
F-XIII	68	F	C	L	A	G	Y	T	T	E	S	S	R	Q	E	E	Q	T	T	C	T	-	T	E	-	G	-	W	S	-	P	E	P	R	C	F	

FIG. 3C

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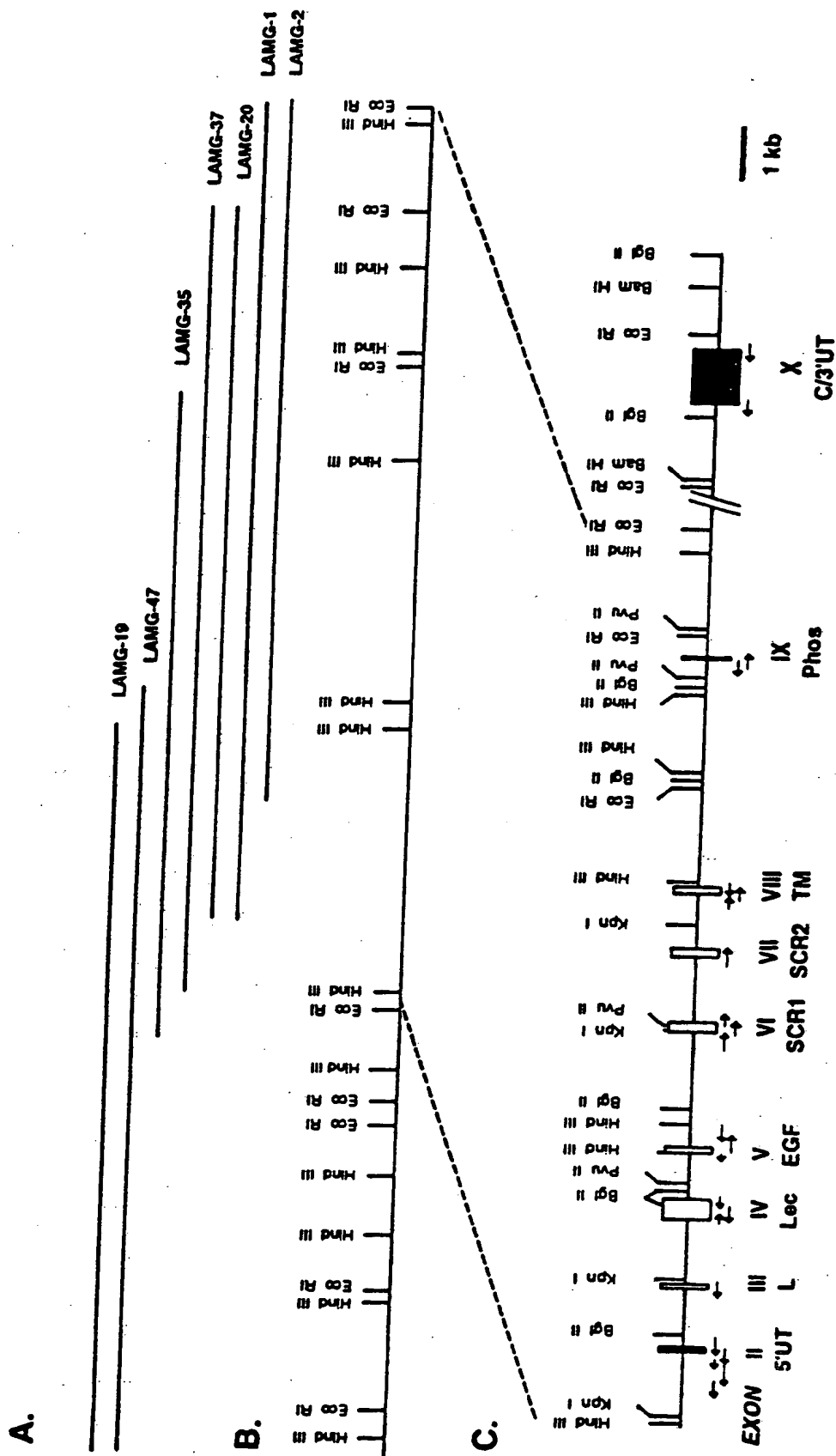


FIG. 4

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EXON II (5' Untranslated Region and Translation Initiation Site)

AGAGAGCTG TTATAAGAT TAACATAT ATAAATATG GCGGTGAGC TTCAGGTTT TTGTTGTTGT TATTATTAT TTCTAARA TTCAATCTG ATTGCAATT
 GACTCATTGA CTCACATCAG TGGGCTCTCC TTTTATTGT CTTTCATCAT ATGGTCTCTA ATTTACATG CAGCTTTATA AACCATCTC ATTTATAGT CCAAGATAT
 TAAGGTACT TGTAGGCTCC CAACCTTACA CCGTGAAAG CTAGAGACA TGGCTCTCT TCAAGGTTA ACTCAGGA GTCCACTA CAGGACGTC CACTAGGTGG
 TGACAGGA AAGACGGAGG TGAGGAGACC GAACGAGTC AGTCCACTG CTTAGCTCTA CTGAGTTT GCAACATCA TAATATGTC TGAATGCG TTTTGATTG
 TAGTATTGC AATTCCAG GCCATTTAC CACAGGTAGC CAGAGTTAG TTTAGCATTT ATCAARAAGA TACGGGAGGG TGGTGGTTA GAGGAGGTG GAGGAGAG
 TGAGGAGGA AGAGGAGAC AAGAACCAA CAARAACAG AACACAGCA ACTAGAGAA GAGGAGCAG GAGGAARAAG AAGAGGAGA AGACAGCA CACATGAG
 TGAGGAGGA GAGGGGTAG GAAGATGCA TAGGAGATG GAGGAGAGG TAGARAGGAG GAGGAGGAG GAGGAGGAG GAGGAGGAG GAGGAGGAG GAGGAGGAG
 TTGCTCAGT GGGGGGCAT AGAGGCCAGT CTAGGAGAGG GGTGGGAAA GAGGAGAGG AGTCCAGGA GAGGAGGAG GAGGAGGAG GAGGAGGAG GAGGAGGAG
 CTGGGTTAG GAATGAAG AAGCAGGC TTCTGTGA CATTACATG AGCTACCTG CAGCAGCA CACTCCCTT GGCAGAGGAC CTGAGACCTT TGCTGAGTA GAACCTCTG
 CAGAGGCCT AATGGGCTG AGAGAGACIA GAGAGGAGC AGCAGAGCC ATG GTGAGCCCTT CAGCCTAARA GACGTTTGA TGCTGAGTA GAACCTCTG
 GGGTTGAGA GCGAGGAGG AAGGATAGGA ATCAGCCCAT TTCAATCTG GTTTTANNTA TAACATTTT CTCAGACCTT CAARAARAGT

EXON III (Leader Domain)

CACTGAGA CTAGCGTAA ATAAATAGA ACARACARAC TGTGATCAG TTCTGATGA AATTGAGT AATTTCATC TATGCTGAG AACCTGTTA CCTCAGACAG
 GGTAGTGA CATATGTTT TTATCTGAT TATTAGAAA GTTGATGCA CCACCTCAA GGTATTAAT GTGTTGTTA AGGTATACA TCTAATATA AATTGATTT
 TCAATTCAG ATA III CCA TGG AAA TGT CAG AGC ACC CAG AGG GAC IIA TGG AAC ATC IIC AAG IIG TGG GGG TGG ACA ATG CIG TGI
 TGT G TATGTTAG AATTTTAT ATCACIAGT CTTTCTACT TATATCAT TTT

FIG. 5A

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EXON IV (Lectin Domain)

CT GGAGTAGTGC TAGGTTCTTT TTACTGTAA CATTATGTAA GTCTGCTAG GTCACTCTA TGCTTGCAG AT IIC CIG GCA CAT CAT GGA ACC GAC TGC
 GGA ACC AAC GGC GAA AII GAG IAT CIG GAG AAG ACI CIG CCI IIC AGI CCI ICI IAC IAC IGG AIA GGA AIC CCG AAG AIA GGA AIA
 AIA IGG ACG IGG GIG GGA ACC AAC AAA ICI CIG ACI GAA GAA GGA GAG AAC IGG GGA GAI GGI GAG CCC AAC AAC AAG AAG AAC AAG
 GAG GAC TGC GIG GAG AIC IAT AIC AAG AGA AAC AAA GAT GCA GGC AAA IGG AAC GAI GAC GGC TGC CAC AAA CIA AAG GCA GGC CIG
 IGI IAC ACA G GTAGGAGTG ACAGACGGC TATGCTGCTT CAGACCTCAGG AAGGCCCCG GTTAGAGAA TACICAGAT TA

EXON V (EGF Domain)

AAATTTTATG CCATATGATT TTTATGCTAT GARATTACCA AATAACCTT TCCIGATTAT TTAAATCATC TCAGACAAA GGTATCTAT GCTAAGAA ATGACTTTGA
 GTACTAATAT GTATACAT TAAATATTT TTTTCTGAC CTCCTTAAG CI ICI IGC CAG CCC IGG ICA IGC AGI GGC CAT GGA IGI GIA
 GAA AIC AIC AAT AAT IAC ACC IGC AAC IGI GAI GIG GGG IAC IAT GGG CCC CAG IGI CAG III G GTAGCTCTT TCCCTTCTT
 TGCCTTCTT TAGGTAAAT CACAGGATC ATTATAGCTT ATCATGAGC TGGTGGAC AAATGATAC TAGCCACTT GAGAAATGGG AGTTTGTAT CAGAAAGCTC
 TGCCTTCTCA ATATGTTAC CTTTCCGTAA AGATTTCATA AGTCAGCATG AGTTTCTAT TCATTCTCA ACAAGTCTT TTGAGTACCA CAGAGACAC AGTGTGGGA
 TAAGCTCTC AGGTTACAA TARGAATTA GCATGGTAA TTCCGCTCT CAGAGAGCTC ACGATCTAAT GAGCTTGTA GATTATTAAG AACTCTAGG TCTGGAGAA
 ACTATGCCAT TTATCATAG GAGGTGAGT TACCAGAA GTATCTTGT TTTTCTCT AGTAGTCTT TTTCTTCTG CAGTCTCCA CACTTACAC ATGTGCTCTG
 TAGCACACTG ACTTGTCTG TGCCTTCTC TCTCATTTG CACATGGCCA AAARACATGT CATCTTAAG ACATTGTCA AAGACAGTTT CTCTAGGA GCTT

EXON VI (SCR 1 Domain)

CCTGA TGATAGTT ATTTCCGAC TAGCTGGC ATTCAGTT ATCCAGTT ACACCTATTT GGCTTAAGG ATTCCTA CAGATAATAC TGAGATAT ATATGAGA
 CTAGTAATG TTTACTAGA ATTTCTGATG AGTCAGGCTT TGTTCTACG TCCTTGACTT ATGCTATG AATTACATTT AGTTCCATA TCAATTGT AAGATACAA
 CAATTTCAAT ATTCCTCTA TATAGATGA GAACGTGAG TTGGAGGCTT CAGTARCT TGTTAAGG CACATGGTA TCAGTGGCA GGGCTAGGAT TCAATCCAG
 GCGTCACTT CACTTACTT TTCCCATAC TGTTCTTTC CCTATTGAG IG AII CAG IGI GAG CCI IIG GAG GGC CCA GAG CIG GGI ACC AIG GAC
 IGI ACI CAC CCI IIG GGA AAC IIG ACC IIC ACC ICA CAG IGI GGC IIC ACC ICI GAA GGA ACA AAC IIA ACI GGG AII GAA
 GAA ACC ACC IGI GGA CCA III GGA AAC IGG ICA ICI CCA GAA CCA ACC IGI CAA G GTAGTACT TCAGCTAGA GOTTGTGTA TGCATCTCIG
 GGCTTACAGT CAGACATTC AGTAGAGTT TGCTGAGAG TCAACTTAG GATCTTAAT TACCTAAT TTTGTTTAC CACTGTGAT GTTCTCAA GGACTTATC

FIG. 5B

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EXON VII (SCR II Domain)

GAGGGTCAC CTTAGCTAGG GCAGCAGCCT GGAGTAGCTA CTCTCTCC CACAGCTTTC ATGCTTCTT TGGCTTCATC TCCTATTCAC CACCCACCAT CATTCACAG
 AATTAAGC CTGGAGGCA TATCAGACT ATGTAGTCA GGCAGCTTIG GCTAATATC CAGAGCTCA GGGGGGCT CTACTCAGAT ATACTGTTT GCTTTTTT
 TTTTCTTT TTTATGAG IG AII CAG TGI CAG CCI CIA ICA GCA GAT IIG GGG AIC AIG AAC TGI AGC CAT CCC CIG CCC AGC
 IIC ACC III ACC TCI GCA TGI ACC IIC AIC TCC ICA GAA GCA ACT GAG IIA AII GGG AAG AAG ACC AII TGI GAA ICA TCI
 GCA AIC TGG ICA AAT CCI AGI CCA AIA TGI CCA A CTAGTAGT TGTCTTGA ACTGA

EXON VIII (Transmembrane Domain)

TATCAGAAC TARGAAGCT TGGGCTGCAG GTGACTCTA GGTCATTTT CAGGACTCT ATGARACCACA AATCTGGCA TTGAGATTCT GTAGGCATTA GACTAGCAG
 GTGGTCACT CTTTGGCTAT GTGTAGACT CATCAGGGC CTTCACATGC CATTTCCTC ATCTGTCAR TGGCATCAT TGGCTACTA CTGGGAGTG TARGAGGAA
 AARGICARA TATCATGAGA TAGACTAAGG AATATATGCT GGIGCTCTA TGTATGTGC CTACTGAT TCTCTTCAG AA IIG GAC AAA AGT IIC ICA AIG
 AII AAG GAG GGT GAT IAI AAT AAC CCC CTC IIC AII CCA GIG CCA GIC AII ACT GCA IIC TCI GGG IIG GCA IIA AIC AII TGG CIG
 GCA AAG ACA IIA AAA AAA G GTATGTAGT TTAATCTAC ATGAARAGCA CACACTTIA AGTGAARAA GAAARAAAAA AGAARCCAC AGGAATTA
 ATGIGATGA TACACACA GCAGGATGCC AAGCT

EXON IX (Phosphorylation Domain)

TAGTTTACA GTATTAGCAG CTGTCCCTCA AGGAGGATC TGCAGGTAGA TGAGATGCAG ATTGGGTGG ATAAACACTT GAATGACATA TGGGTCTTG CCACAGGCA
 ATTTAGCAAT TCTGTCTTCT TGAGTAGCAC GGGATGGAA TGGACCTCA GAGGGCATCT GCATCAGCAT GTCTGTCTG TATTAGTCT TACCCTGTT TATTAGCCA
 GTTCTCAAA TCTCTCTTGA CACAGTAGG GTCCACCTAA CAATACCTA ATATACTTCA AAGACAGCT TTGAGAGTGG GAGCTTCTT TCTCCCTAC TTGAARACT
 TTAATTTGC TAAATTTGC TAAATCTT TCTCTATT TCTATTTCAG GC AAG AAA TCC AAG AAG AG GTAGTTTIA TTAGTGGCA GAGTTTCCA
 CATCTCTGA TTAATCTCT ACTTCTTAA GTACTTCTGC TCTAGCTAGA CACATACCCA TAGTAGTTAT TACTGGTCT ATCAATGACA GATAGG

EXON X (Cyt plasmic Tail and 3' Untranslated Region)

ATAGCATC ACTAAGAGC TTGTAGGG TGCAGATCT CAGGCTCCAC TCAGACCTAC TGAATCAGAG TCTGCTTTT AACACCATCT CTGAGTGGTA AGACATGAA
 ATCTGAGAA GTCTGCTAC TAGGTTCG TTAATTTGT TCACTTTCAG AGGTTCTTAA AGCTTGGCT CTGTCTGAG ATTTCAGCT GAAGCATTT CTTGCTCTT
 CTCTCATCT CTATGATA TTTACTTTA CTACTACAC TCCAGTTT GCATTTTAA AACCTTAT AICITTTT TTTCTTCAG I AIG AAT GAC CCA
 IAI IAA ATCCCTTIG GAGGAGAA ATCTTGGAA TACTTGGAA TACTTGGAA TACTTGGAA TACTTGGAA TACTTGGAA TACTTGGAA TACTTGGAA TACTTGGAA
 CAATTTTGA TTTGAATAAA CCTTCTTT TATGTTTGA CTAGTTTGG CTAGTTTGA TTTGAAT TATCATATG TTTTGTCTT TCCCTAGTGA GTTACATGCT
 CCTCAGGGC AGGTTTGTG TCAATCTCT GAGTATCTA GTCTATCT TCACTTCA TAAATGATG TTAATTAAT TCAATTAAT TCAATTAAT TCAATTAAT TCAATTAAT
 TAAATTTAG CATCTCTT TCTATATGC ATCTGATGAC CTGGGTGAGA TATCACTTAA TGTACACAGC TGAATC

FIG. 5C

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/08467

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 31/715, 37/00, 39/395, 49/00; C12Q 1/02; G01N 33/567

US CL : 424/1.1, 9, 85.8, 85.91; 435/7.24, 7.8, 29; 436/503; 530/402

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.1, 9, 85.8, 85.91; 435/7.24, 7.8, 29; 436/503; 530/402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
<u>X</u> <u>E</u> <u>Y</u> A.	Science, vol. 258, issued 06 November 1992, L.A. LASKY, "Selectins: interpreters of cell-specific carbohydrate information during inflammation", pages 964-969. See page 967 cols. 1-3 and page 968 cols. 1 and 3.	6,8-9,11-12,17-18, <u>21,23-25</u> 1-4,6-9,11-12,14-18, <u>20,23</u> 26
<u>X</u> <u>Y</u>	Nature, vol. 349, issued 17 January 1991, T.A. SPRINGER ET AL, "Sticky sugars for selectins", pages 196-197. See page 196, cols. 1-2 and page 197 cols. 2-3.	<u>17-18,21,23</u> 20
X	Cell, vol. 63, issued 30 November 1990, B.K. BRANDLEY ET AL, "Carbohydrate ligands of the LEC cell adhesion molecules", pages 861-863. See page 861, col. 1.	21
Y	US, A, 5,002,873 (ST. JOHN ET AL) 26 March 1991. See col. 3 lines 1-36; col. 4, lines 18-33 and 49-55.	1-4,6-9,11-12,14-18, 20,23

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 December 1992

Date of mailing of the international search report

08 JAN 1993

 Name and mailing address of the ISA/
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Authorized officer

DAVID SAUNDERS, PRIMARY EXAMINER

Facsimile N . NOT APPLICABLE

Telephone N . (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application N .
PCT/US92/08467

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- I. Claims 1-10, drawn to inflammation detection in vivo using labelled LAM-1, classified in class 424/9.
- II. Claims 11-16 and 24-25, drawn to body treatment methods with LAM-1, classified in class 514/2.
- III. Claims 17-20 and 23-25, drawn to body treatments with LAM-1 antagonists, classified in class 514/54.
- IV. Claims 21-22, drawn to assays to identify LAM-1 ligands, classified in class 435/7.24.
- V. Claim 26, drawn to cell adhesion assays, classified in class 435/29.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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